Amendments to the Specification

1. Please replace the paragraph beginning at page 1, line 1, with the following rewritten paragraph:

> ~~Novel~~ Type III Secretion Pathway in Aeromonas salmonicida, and uses therefor

2. Please delete the following paragraph at page 15, line 29 that reads "(see Appendix A)."

3. Please delete the paragraphs on page 21 and 22 of the specification that comprise "Appendix A."

4. Please add the following paragraphs (formerly comprising Appendix A) into the specification immediately following the heading "Recombinant AcrV Vaccine Trial" on page 15, line 28:

Materials
VACCINE FORMULATIONS:
1. The AcrV vaccine was formulated using recombinant, Histidine-tagged AcrV resuspended in 10 mM phosphate buffer, pH 7.0, to 112.5 µg/mL. Four parts of this protein solution were mixed with one part oil adjuvant for a final AcrV concentration of 90 µg/mL. The dose for testing was 0.1 mL, or 9 µg/fish.
2. The commercial comparator vaccine was serial 4-13 of the vaccine **MultiVacc4** (Bayotek International Ltd.)
3. The placebo (control) vaccine consisted of phosphate buffered saline (PBS) (10 mM phosphate, 150 mM NaCl, pH 7.2).
4. All vaccines were maintained at 4°C until use.

Methods

TRIAL DESIGN:

Fish (rainbow trout *Oncorhynchus mykiss*) that have been determined to be pathogen free and are at least 15g in size are held for at least one-week pre vaccination for acclimation purposes. During the acclimation period the fish are offered 1% body weight in salmonid fish food every day, however they are denied food 24 hours pre and post-vaccination.

At least 50 fish are vaccinated 0.1 mL of AcrV vaccine via intra-peritoneal (IP) injection, or 0.2 mL of the commercial vaccine **MultiVacc4.** At the same time a group of at least 50 fish from the same stock are mock vaccinated with 0.1 mL of **PBS**. Vaccinated fish are then held for a period of at least 350-degree days to allow specific immune response generation in an acclimation tank with a continuous flow of water at a temperature of 12-13°C. The fish are offered 1% body weight in salmonid fish food daily until 24 hours pre- challenge and post-challenge.

After at least 350-dgree days post vaccination 50 fish per group were challenged by IP injection with a pre-determined concentration of virulent *Aeromonas salmonicida*. The dosage depends on the source of the fish and the water temperature (this is determined empirically immediately prior to challenge of test fish). The identical procedure is performed with the placebo vaccinated control fish. The fish are observed daily for mortality for 21 days post challenge and the cause of mortality assessed and examined to ensure that mortality is attributed to the challenge organism. After 24 hours post-challenge the fish are again offered 1% body weight in salmonid fish feed daily. Tanks are maintained with a continuous flow of water at a temperature of 12-13°C. For a challenge series to be considered satisfactory; all challenge groups must meet the following criteria:

1. At least 70% of the non-immunized controls must die within 21 days of challenge.
2. A relative percent survival (RPS) of no less than 25% must be achieved for the challenge disease before a vaccine is considered even partially efficacious for this disease.

RPS = [1-(% mortality vaccinates/% mortality controls)] x 100

Developed from: The Rules Governing Medicinal Products in the European Union, Volume VII, Guidelines for the testing of veterinary medicinal products. 1994. Specific Requirements for the Production and Control of Live and Inactivated Vaccines Intended for Fish. Section 3.2. Potency.

RESULTS

Group	% Mortality	RPS
PBS	82	-
AcrV	49	40
MultiVacc4	30	63

There was a strong challenge with 82% control mortalities.

5. Please replace the paragraph at beginning at page 8, lines 24 to 34 of the specification with the following rewritten paragraph:

Sequence alignment and editing were performed by using the software Sequencher (Gene Codes Corporation, Ann Arbor, MI, USA). Comparisons of DNA sequences and their deduced amino acid sequences with EMBL/GenBank and NBRP databases were performed using the programs BLASTN, BLASTX and BLASTP (Altschul et al., 1990). Potentially antigenic segments of AcrV were determined using the software *ProtScale* ~~(http://www.expasy.ch/cgi-bin/protscale.pl)~~ (Bairoch et al., 1995) and the software *Coils output* ~~(http://www.ch.embnet.org/software/COILS_form.html)~~ (Lupas et al., 1991). The molecular masses of the protein and its theoretical isoelectric pH (pI) were calculated by using ProtParam tool ~~(http://www.expasy.ch/tools/protparam.html)~~ (Gill and von Hippel, 1989). Transmembrane prediction of the protein were made by using

Tmpred ~~(http://www.ch.embnet.org/software/TMPRED_form.html)~~
(Hofmann and Stoffel, 1993).

6. Please replace the paragraph appearing at lines 11 to 16 of page 1 with the following rewritten paragraph:

> *Aeromonas salmonicida*, a Gram-negative, facultatively anaerobic, non-motile, rod shaped bacterium, growing at temperatures around 20°C, is the etiological agent of furunculosis in salmonids, causing most severe economic losses in production farms of salmon and trout. The disease is ~~characterised~~ characterized in the sub-acute or chronic form by the presence of haemorrhagic necrotic lesions in the gills, gut and muscle, while in the acute form fish die apparently from toxaemia without showing particular external signs.

7. Please replace the paragraph appearing at lines 16 to 24 of page 12 with the replacement paragraph below, in which the applicant has replaced the abbreviation "mio" with the word "million" in compliance with the Examiner's request:

> RTG-2 fish cells were grown as described above. Two days before infection 20 ~~mio~~ milion of trypsinized RTG-2 fish cells were seeded into 24 well culture plates (1.9 cm^2) (Techno plastic products AG, Trasadingen, Switzerland). Rabbit antiserum directed against AcrV as well as control preserum were decomplemented for 30 min at 56°C. A fresh culture of *A. salmonicida* (at end exponential growth phase) was washed and resuspended in PBS pH 7.4 and mixed with either preserum or anti AcrV antiserum at a ratio of 1:1, 1:10, 1:100, 1:1000 or 1:10'000. Bacteria were incubated with the serum at 18°C for 30 min. The opsonized bacteria were added to the fish cells in a ratio of 20:1 or 2:1 (bacteria/fish cells). After 21 hrs of infection at 15°C the fish cells were photographed as described before and inspected for morphological changes.

8. Please replace the paragraph appearing at lines 1 to 7 of page 5 with the replacement paragraph below, in which the applicant has cured the typographical error in the parenthetical note:

> The least conserved protein encoded on the cloned and analyzed segment is AcrV, which shows only 35% identical aa to PcrV of *P. aeruginosa* and 37% identity to LcrV of *Y. enterocolitica*. The main role of LcrV and PcrV, and accordingly also of AcrV, is assumed to be involved in sensing the bacterium-host interactions (Sawa et al., 1999; Bergman et al., 1991). We therefore interpret the significantly higher dissimilarity between AcrV and LcrV or PcrV, compared to the other gene products of the type III secretion locus ~~(Table 2)~~ (Table 3), to be due to the host specificity which seems to be determined by AcrV, LcrV or PcrV.

9. Please delete Table 2 on page 24 of the specification, and replace it with the replacement Table 2

Table 2.
Oligonucleotide primers

Name	Sequence[a] 5' to 3'	~~Position~~ Residue Nos. of SEQ ID NO:10 [b]	Annealing Temp °C
AslcrD-L[c]	GCCCGTTTTGCCTATCAA	1159-1176	60
AslcrD-R[c]	GCGCCGATATCGGTACCC	2028-2011	60
AcrV-L[c]	TTCGTCGGCTGGCTTGATGT	4144-4163	58
AcrV-R[c]	GAACTCGCCCCCTTCCATAA	4734-4715	58
AsacrVt-L[d]	gggaattcGATGAGCACAATCCCTGACTAC (SEQ ID NO: 11)	4104-4125	57
AsacrVt-R[d]	atgcggccgcAAATTGCGCCAAGAATGTCG (SEQ ID NO: 12)	5188-5169	57
AsacrVN'-R[d]	tcgcggccgcACCCTTTACGCTGATTGTC (SEQ ID NO: 13)	4555-4537	57
AsacrVC'-L[d]	cggaattcGTTGCGGGATGAGCTGGCAG (SEQ ID NO: 14)	4554-4573	57
AsacrVC'-R[d]	tcgcggccgcACTCGGCTTCTATGCCACTC (SEQ ID NO: 15)	4987-4968	57

[a] Lowercase letters indicate nucleotides added to create restriction enzyme recognition sites (underlined) for cloning.

[b] Based on nucleotide sequence of *A. salmonicida* JF2267

[c] Primer used for gene probe preparation

[d] Primer used for amplification of gene *acrV*, *acrV*-N, and *acrV*-C respectively

10. Please delete the Sequence Listing, and replace it with the revised Sequence Listing submitted herewith.